PATENT
Docket No.: **176/60792 (6-11415-868)**

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicant(s) :	Mahin D. Maines	)	
Serial No. :	09/606,129	)	Examiner: D. Ramirez
Cnfrm. No. :	5529	)	Art Unit: 1652
Filed :	June 28, 2000	)	
For :	BILIVERDIN REDUCTASE FRAGMENTS AND VARIANTS, AND METHODS OF USING BILIVERDIN REDUCTASE AND SUCH FRAGMENTS AND VARIANTS	)	

DECLARATION OF MAHIN D. MAINES UNDER 37 C.F.R. § 1.132

1. I am the inventor of the above-identified application.

2. I am currently Professor of Biochemistry and Biophysics at the University of Rochester Medical Center, Rochester, NY.

3. I received a B.A. in Biology from Ball State University in 1964, an M.S. in Chemistry from Ball State University in 1967, and a Ph.D. in Pharmacology from the University of Missouri in 1970. A major focus of my career has been involved with biliverdin reductase, its activities, its properties, and how the protein interacts with other cellular components to regulate cell activities. Since 1981, I have published over twenty-four articles concerning different aspects of biliverdin reductase.

4. I am presenting this declaration to demonstrate that the structure and function of biliverdin reductase ("BVR") proteins are highly conserved among mammalian BVR and, therefore, results achieved with human and/or rat BVR are predictive of results that can be achieved with other mammalian BVR.

5. The present application identifies two human BVR amino acid sequences (SEQ ID NO: 1 and SEQ ID NO: 3) and a single rat BVR amino acid sequence

(SEQ ID NO: 4). The two human BVR sequences are 99 percent identical. The human BVR of SEQ ID NO: 1 and the rat BVR of SEQ ID NO: 4 are 82 percent identical.

6. Mammalian BVR proteins are characterized by a number of shared structural features. As identified in the present application, human BVR of SEQ ID NO: 1 is characterized by the following structural features: a basic N-terminal domain characterized by the sequence ERK at residues 6 to 8; a hydrophobic domain characterized by the sequence FGVVVV at residues 9 to 14 (which matches the consensus sequence of SEQ ID NO: 6); a nucleotide binding domain characterized by the sequence GVGRAG at residues 15 to 20 (which matches the consensus sequence of SEQ ID NO: 7); an oxidoreductase domain characterized by the sequence AGKHVLVE at residues 90 to 97 (SEQ ID NO: 8 as corrected); a leucine zipper characterized by the sequence $LX_6LX_6KX_6LX_6L$ at residues 129 to 157 (SEQ ID NO: 9); several kinase motifs characterized by the sequence SRR at residues 44 to 46 (SEQ ID NO: 10), KGS at residues 147 to 149 (SEQ ID NO: 11), and FGF at residues 162 to 164 (which matches the consensus of FGX, SEQ ID NO: 12); a nuclear localization signal characterized by the sequence GLKRNRY at residues 222 to 228 (SEQ ID NO: 13); a myristylation site characterized by the sequence PGLKR at residues 221 to 225 (SEQ ID NO: 14); a zinc finger domain characterized by the sequence $HCX_{10}CC$ at residues 280 to 293 (SEQ ID NO: 15); a protein kinase C ("PKC") enhancing domain characterized by the sequence KKRILHC at residues 275 to 281 (which matches the consensus of SEQ ID NO: 16); and a PKC inhibiting domain characterized by the sequence QKYCCSRK at residues 290 to 296 (which matches the consensus of SEQ ID NO: 17). I have since identified additional kinase motifs within the human BVR sequence, including GRAGSVRM at residues 17 to 24 which conforms to the XRXXSXRX motif (Kemp et al., "Protein Kinase Recognition Sequence Motifs," *Trends in Biological Sciences* 15(9):342-346 (1990) (copy attached hereto as **Exhibit 1**), and YMKM at residues 198 to 201 which conforms to the YMXM motif (*see* Shoelson et al., "YMXM Motifs of IRS-1 Define Substrate Specificity of the Insulin Receptor Kinase," *Proc. Natl. Acad. Sci. USA* 89:2027-2031 (1992) (copy attached hereto as **Exhibit 2**). The rat BVR sequence of SEQ ID NO: 4 contains an identical hydrophobic domain, an identical nucleotide binding domain, an identical oxidoreductase domain, a conserved leucine zipper domain (i.e., with any residue variations being between L and K residues), identical or conserved kinase motifs, an identical nuclear localization signal, an identical myristylation site, a conserved zinc finger domain, a conserved PKC enhancing

domain, and a conserved PKC inhibiting domain. Based on the shared or conserved structural features between the human and rat BVR sequences, one of ordinary skill in the art would expect other mammalian BVR sequences to share these same identical or conserved structural features.

7. The reasonableness of the expectation of shared structural features, based on a comparison of human and rat BVR sequence, is confirmed by the alignment of human and rat BVR sequences with the mouse and pig BVR sequences, which have subsequently been obtained. The mouse BVR sequence, obtained in my laboratory, is reported at Genbank Accession NP_080954, a copy of which is attached hereto as **Exhibit 3**. The pig BVR was also obtained in my laboratory using total RNA isolated from *Sus scrofa* muscle tissue. The RNA was used to make cDNA in a reverse transcription reaction with iScript cDNA synthesis kit (BIO-RAD, #170-8890). 2 µl of the cDNA prep was used to amplify the biliverdin reductase open reading frame with human specific full-length primers under the following conditions: initial denature 94°C, 2 min; 40 cycles: denature temperature 94°C for 30 sec, annealing temperature 65°C for 30 sec, extension temperature 72°C for 1 min; and final extension 72°C, 10 min. The PCR product was purified with QIAquick gel purification kit (Qiagen, #28704) and used for direct sequencing with gene-specific primers giving overlapping fragments with BigDye terminator mix (version 3.1). Independent purification of total RNA from another source of *Sus scrofa* muscle tissue, cDNA synthesis, PCR amplification, and gel purification was done as described above. The PCR product was used to clone into the pETBlue vector plasmid (Novagen, #70599-4). Recombinant DNAs were checked for insert and orientation using PCR analysis with gene specific primers and restriction endonucleases *Xba*I and *Eco*RI. One of the plasmids pETBlue/SsBVR-3 was used for sequencing analysis. Both independent sets of sequences, from cDNA amplicon and from cloned fragment, were in perfect match with each other. A copy of the pig BVR cDNA and amino acid sequences is attached hereto as **Exhibit 4**. The mouse and pig BVR amino acid sequences were aligned with the human and rat BVR amino acid sequences using the ClustalW alignment program set on its default settings. A copy of the alignment of these four mammalian BVR sequences is attached hereto as **Exhibit 5**.

8. The mouse BVR sequence is about 81 percent identical to the human BVR sequence of SEQ ID NO: 1. Based on the alignment of Exhibit 3, one of ordinary skill

in the art would conclude that mouse BVR, when compared to the human BVR of SEQ ID NO: 1, contains an identical hydrophobic domain, an identical nucleotide binding domain, an identical oxidoreductase domain, a conserved leucine zipper domain (i.e., with any residue variations being between L and K residues), identical or conserved kinase motifs, a conserved nuclear localization signal, an identical myristylation site, a conserved zinc finger domain, an identical PKC enhancing domain, and a conserved PKC inhibiting domain. This high structural conservation, particularly within previously identified functional domains of the protein, indicates that the proteins are functionally quite similar.

9. The pig BVR sequence is about 98 percent identical to the human BVR sequence of SEQ ID NO: 1. Based on the alignment of Exhibit 3, one of ordinary skill in the art would conclude that the pig BVR, when compared to the human BVR of SEQ ID NO: 1, contains an identical hydrophobic domain, an identical nucleotide binding domain, an identical oxidoreductase domain, a conserved leucine zipper domain (i.e., with any residue variations being between L and K residues), identical or conserved kinase motifs, an identical nuclear localization signal, an identical myristylation site, a conserved zinc finger domain, an identical PKC enhancing domain, and an identical PKC inhibiting domain. This high structural conservation, particularly within previously identified functional domains of the protein, indicates that the proteins are functionally quite similar.

10. In addition, based on the known biochemical pathways shared by mammalian BVR proteins, one of ordinary skill in the art would have expected the results achieved with one mammalian BVR to be consistent with other mammalian BVR. Prior to the present application, it was widely believed that BVR was a general housekeeping enzyme that was conserved among mammals, catalyzing the NADPH-dependent reduction of biliverdin to produce bilirubin. In particular, Noguchi et al., "Purification and Properties of Biliverdin Reductase from Pig Spleen and Rat Liver," *J. Biochem.* 86(4):833-848 (1979)("Noguchi")(copy attached as **Exhibit 6**) reports that purified pig and rat BVR has both NADH- and NADPH-dependent activities in converting biliverdin to bilirubin, with the NADH-dependent activity being optimal at pH 6.9 and the NADPH-dependent activity being optimal at pH 8.5. Noguchi also indicates that both systems are inhibited by bilirubin, but inhibition of the NADPH-dependent activity was more pronounced. In addition, Noguchi reports that the NADPH-dependent activity for biliverdin had a K_m of 0.3 μM whereas the

NADH-dependent activity for biliverdin had a K_m of 1-2 μM. Rigney et al., "The Reaction Mechanism of Bovine Kidney Biliverdin Reductase," *Biochim. Biophys. ACTA* 957:237-242 (1988)(copy attached as **Exhibit 7**) reports that purified bovine BVR has both NADH- and NADPH-dependent activities in converting biliverdin to bilirubin, with the NADH-dependent activity being optimal at pH between 6 and 7 (depending on the buffer system utilized) and the NADPH-dependent activity being optimal at pH 8.5. Rigney et al., "The Kinetics of Ox Kidney Biliverdin Reductase in Pre-steady State: Evidence That the Dissociation of Bilirubin is the Rate-determining Step," *Biochem J.* 259:709-713 (1989)(copy attached as **Exhibit 8**) confirms that the broad features of the reaction mechanism for NADPH- and NADH-dependent activities are the same, with BVR activity exhibiting a pH-dependent burst in the rate of conversion of biliverdin to bilirubin followed by a steady-state rate. As addressed in the present application, at Example 1, human BVR shares the property of dual co-factor activity using NADPH and NADH. In addition to the conserved activity among mammalian BVR, Rigney et al., "Some Physical and Immunological Properties of Ox Kidney Biliverdin Reductase," *Biochem J.* 255:431-435 (1988)("Rigney III")(copy attached as **Exhibit 9**) reports at Table 2 and page 435 (first column) that antibodies raised against ox BVR were able to immunoprecipitate BVR from numerous mammals, including pig, guinea pig, mouse, rat, hamster, fox, wallaby, and human. All of the foregoing confirms that those persons of skill in the art believed BVR to be functionally well-conserved among mammals.

11. Based upon the high degree of structural similarity of the three BVR proteins identified in the present application, addressed in paragraphs 5 and 6 above, as confirmed by their high degree of structural similarity with mouse and pig BVR sequences as addressed in paragraphs 7-9 above, and the functional similarity of many mammalian BVR proteins as identified in paragraph 10, persons of skill in the art would have expected results achieved with any one mammalian BVR protein to be achievable with other mammalian BVR proteins.

12. I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the application or any patent issuing thereon.

Date: 3/2/04

Mahin D. Maines

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